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hereby declare:

1. That I am a citizen of Japan.
2. That I am well acquainted with both the Japanese and English languages.
3. That I am the translator of the document (Application claiming the priority of Japanese Patent Application No. 2002-242259 filed on August 22, 2002) and certify that the following is a true translation to the best of my knowledge and belief.

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[Item] Claims 1

[Item] Drawings 1

[Item] Abstract 1

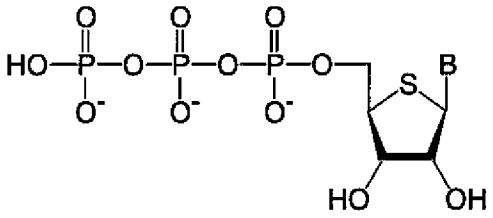
[Necessity of Proof] Yes

Description

4'-THIONUCLEOTIDES

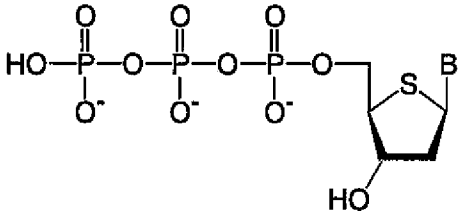
Claims

1. A compound of formula I:



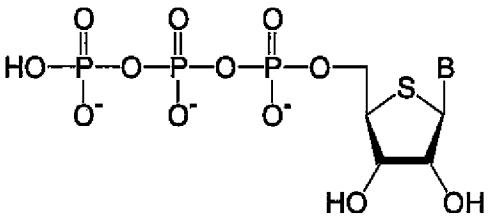
[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, uracil and hypoxanthine].

2. A compound of formula II:



[wherein B' is a nucleobase selected from the group consisting of adenine, guanine, cytosine, thymine, uracil and hypoxanthine].

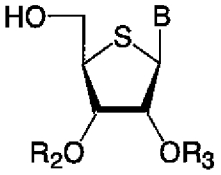
3. A method for synthesizing a compound of formula I:



[wherein B is a nucleobase selected from the group consisting

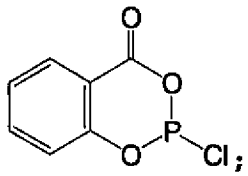
of adenine, guanine, cytosine, uracil and hypoxanthine],

said method comprising reacting a compound of formula III:



[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, uracil and hypoxanthine, and each of R_2 and R_3 is, independently a protecting group of a hydroxyl group]

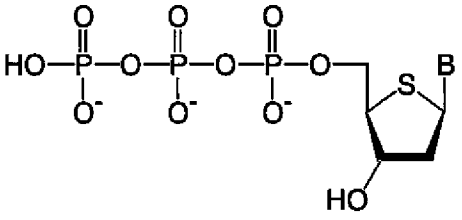
with a compound of formula IV:



reacting the resulting intermediate with pyrophosphoric acid; and

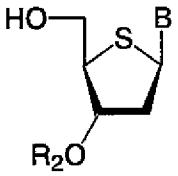
conducting iodo-oxidation, hydrolysis and deprotection to obtain the compound of formula I.

4. A method for synthesizing a compound of formula II:

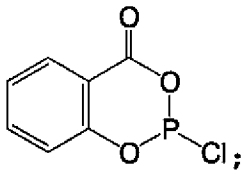


[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, thymine, uracil and hypoxanthine],

said method comprising reacting a compound of formula V:



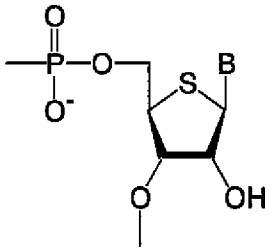
[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, thymine, uracil and hypoxanthine, and R_2 is a protecting group of a hydroxyl group] with a compound of formula IV:



reacting the resulting intermediate with pyrophosphoric acid; and

conducting iodo-oxidation, hydrolysis and deprotection to obtain the compound of formula II.

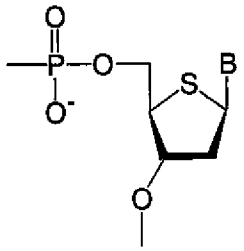
5. A process for producing an oligonucleotide containing at least one nucleoside unit of formula VI:



[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, uracil and hypoxanthine] comprising conducting RNA chain elongation reaction with RNA synthetase in the presence of the compound of claim 1 or the

compound produced by the method according to claim 3.

6. A process for producing an oligonucleotide containing at least one nucleotide unit of formula VII:



[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, thymine, uracil and hypoxanthine]

comprising conducting DNA chain elongation reaction with DNA synthetase in the presence of the compound of claim 2 or the compound produced by the method according to claim 4.

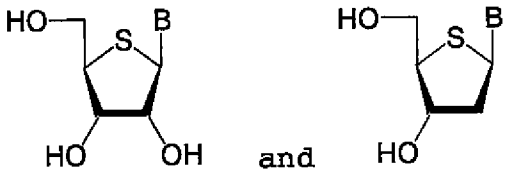
Technical Field

The present invention relates to nucleotide analogues and a process for producing the same. More specifically, the invention relates to a 4'-thioribonucleotide and a 4'-thio-2'-deoxynucleotide, a process for producing these nucleotide analogues, and a process for producing oligonucleotides using these nucleotide analogues.

Background Art

4'-Thionucleoside is a general term of nucleosides in which an oxygen atom of a furanose ring is substituted with

a sulfur atom.



RNA or DNA containing a 4'-thioribonucleoside or a 4'-thio-2'-deoxyribonucleoside is suggested to be useful as an investigation reagent and a diagnostic or therapeutic agent, because it shows resistance to various nucleases.

Bellon et al. (Biochem. Biophys. Res. Comm., 1992, 184, 797-803) describe synthesis of oligodeoxynucleotides containing 1-(4-thio-β-D-ribofuranosyl)thymine. Bellon et al. (Nucleic Acids Res., 1993, 21, 1587-1593), Leydier et al. (Antisense Res. Rev. 1995, 5, 167-174) and Leydier et al. (Nucleosides Nucleotides, 1995, 14, 1027-1030) describe that a 4'-thio-β-D-oligoribonucleotide has high nuclease resistance. Dukhan et al. (Nucleosides Nucleotides, 1999, 18 1423-1424) describe synthesis of oligonucleotides containing four types of 4'-thioribonucleosides.

Bellon et al. (Nucleic Acids Res., 1993, 21, 1587-1593) have examined resistance to a degradation enzyme of RNA solely comprising 4'-thiouridine, and have reported that 4'-$^{S}U_6$ shows by far higher resistance to various degradation enzymes than native-type U_6.

Meanwhile, several synthesis examples of deoxyribonucleosides have been reported. Hancox et al.

(Nucleic Acids Res., 1993, 21, 3485-3491) describe synthesis of 2'-deoxy-4'-thiothimidine and oligodeoxyribonucleotide containing the same. Boggon et al. (Nucleic Acids Res., 1996, 24, 951-961) describe a structure of a synthetic DNA oligomer containing 4'-thio-2'-deoxythimidine. Jones et al. (Nucleic Acids Res., 1996, 24, 4117-4122) and Jones et al. (Bioorg. Med. Chem. Lett., 1997, 7, 1275-1278) describe a synthetic DNA oligomer containing 4'-thio-2'-deoxynucleotide. Kumar et al. (Nucleic Acids Res., 1997, 25, 2773-2783) describe a synthetic DNA oligomer containing 4'-thio-2'-deoxycytidine.

However, all of these oligoribonucleotides and oligodeoxyribonucleotides are produced by chemical synthesis. By this method, long-chain oligonucleotides can hardly be obtained, and the cost is high.

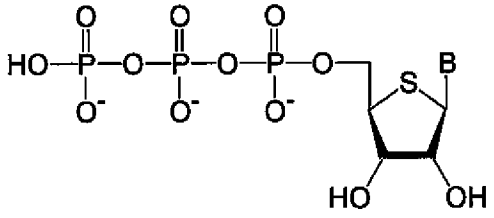
For synthesizing oligonucleotides from 4'-thionucleosides using an enzyme such as RNA polymerase, DNA polymerase or reverse transcriptase, 4-thionucleosides have to be triphosphorylated. Alexandrova et al. (Antiviral Chemistry & Chemotherapy, 1995, 7, 237-242) describe synthesis of 4'-thio-5-ethyl-2'-deoxyuridine 5'-triphosphate and recognition of this compound by DNA synthetase. However, 4'-thiodeoxyribonucleoside triphosphate or 4'-thioribonucleoside triphosphate with other nucleobase has not been obtained so far. This is presumably because stereoselective synthesis is difficult.

Thus, a novel improved method for synthesizing 4'-thionucleotides which can be recognized and elongated by an enzyme such as RNA polymerase or DNA polymerase is needed in the art.

Accordingly, the invention aims to provide novel nucleoside triphosphates, a method for synthesizing the same, and a process for producing oligonucleotides using these nucleoside triphosphates.

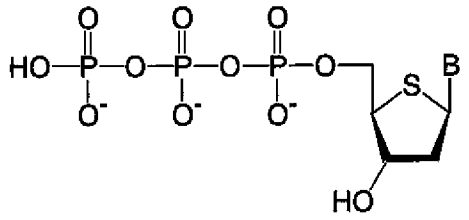
Disclosure of the Invention

The invention provides a 4'-thioribonucleotide represented by formula I:



[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, uracil and hypoxanthine].

The invention also provides a 4'-thio-2'-deoxynucleotide represented by formula II:

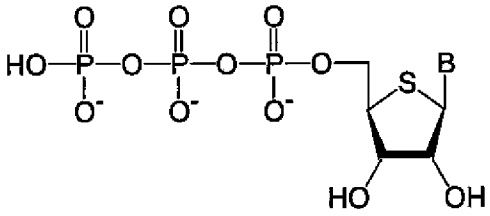


[wherein B' is a nucleobase selected from the group consisting

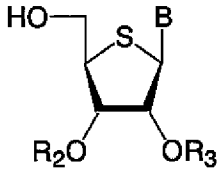
of adenine, guanine, cytosine, thymine and hypoxanthine].

Since 4'-thionucleotides of the invention can be recognized and elongated with an enzyme such as RNA polymerase or DNA polymerase, they are useful as a monomer unit for synthesizing RNA or DNA having resistance to nucleases.

In another aspect, the invention provides a method for synthesizing a 4'-thioribonucleotide represented by formula I:

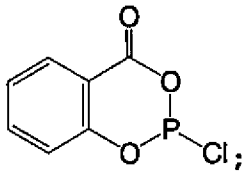


[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, uracil and hypoxanthine] comprising reacting a compound of formula III:



[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, uracil and hypoxanthine, and each of R_2 and R_3 is independently a protecting group of a hydroxyl group]

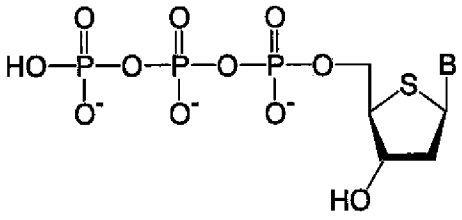
with a compound of formula IV:



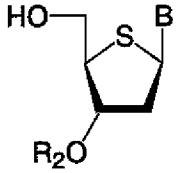
reacting the resulting intermediate with pyrophosphoric acid; and

conducting iodo-oxidation, hydrolysis and deprotection.

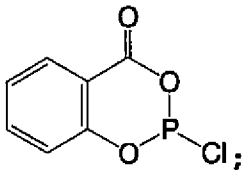
In still another aspect, the invention provides a method for synthesizing a 4'-thio-2'-deoxynucleotide represented by formula II:



[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, thymine and hypoxanthine] comprising reacting a compound of formula V:



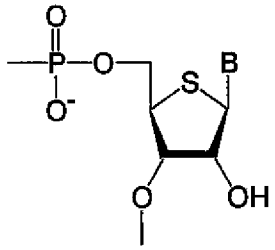
[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, thymine and hypoxanthine, and R_2 is a protecting group of a hydroxyl group] with a compound of formula IV:



reacting the resulting intermediate with pyrophosphoric acid; and

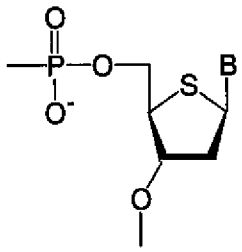
conducting iodo-oxidation, hydrolysis and deprotection.

In another aspect of the invention, the invention provides a process for producing an oligonucleotide containing at least one nucleoside unit of formula VI:



[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, uracil and hypoxanthine] comprising conducting an RNA chain elongation reaction with RNA synthetase in the presence of the 4'-thioribonucleotide of the invention. The RNA synthetase includes RNA polymerase.

In still another aspect of the invention, the invention provides a process for producing an oligonucleotide containing at least one nucleotide unit of formula VII:



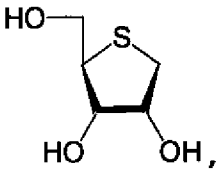
[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, thymine and hypoxanthine] comprising conducting a DNA chain reaction with DNA synthetase in the presence of the 4'-thio-2'-deoxynucleotide of the invention. Examples of the DNA synthetase include DNA polymerase, reverse transcriptase and terminal deoxynucleotide transferase.

Since RNA or DNA containing a 4'-thioribonucleoside or a 4'-thio-2'-deoxyribonucleoside produced by the process of the invention shows resistance to various nucleases, it is useful as an investigation reagent and as a diagnostic or therapeutic agent. According to the invention, since an oligonucleotide can be synthesized by means of an enzyme, an oligonucleotide having longer chain can easily be produced in comparison to the conventional chemical synthesis methods.

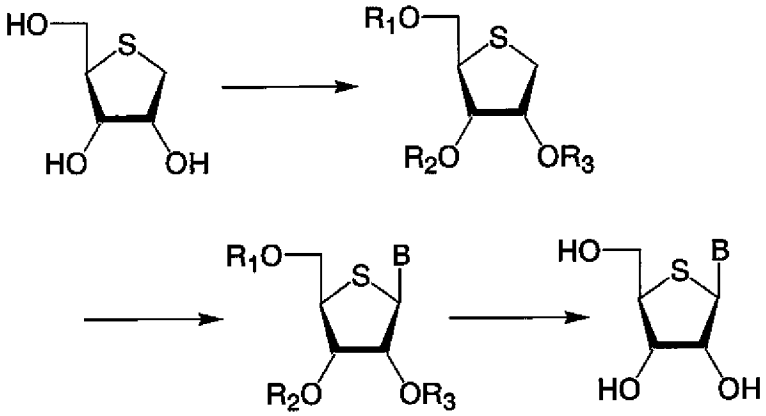
Detailed Description of the Invention

The nucleoside triphosphate of the invention can be produced by starting from known 4'-thio sugar, stereoselectively introducing a nucleobase into the sugar and then selectively introducing a phosphate group at the 5'-position.

4'-Thiouridine can be obtained by appropriately protecting hydroxyl groups at the 2-, 3- and 5-positions of a 4'-thio sugar



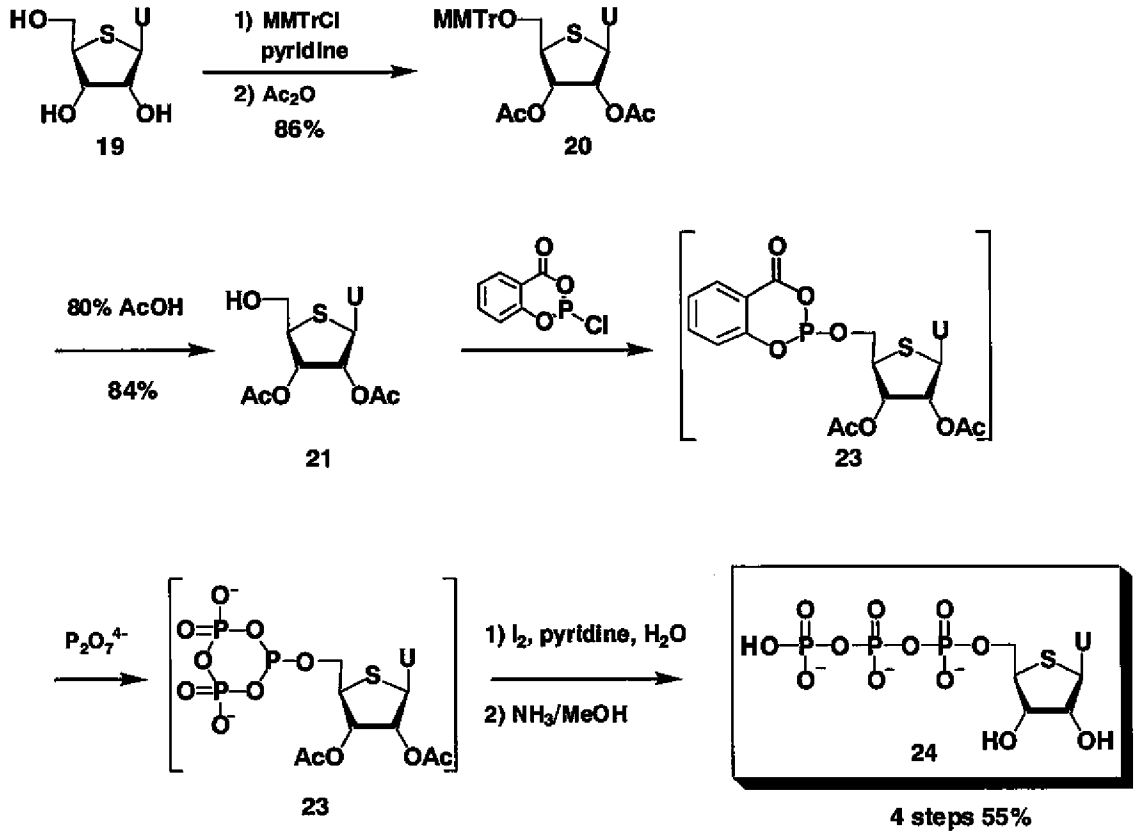
and stereoselectively introducing a nucleobase using a Pummerer reaction.



[wherein each of R_1, R_2 and R_3 independently represents a hydroxyl protecting group, R_1 and R_2 or R_2 and R_3 may together be a bifunctional hydroxyl protecting group].

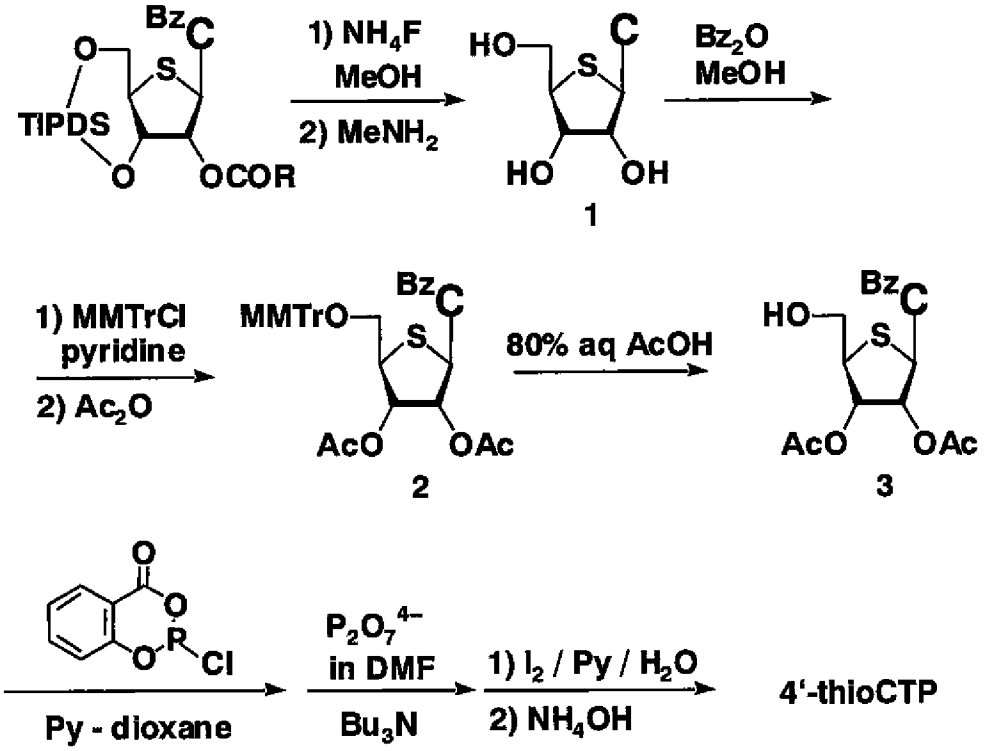
When R_3 is an acyl protecting group having an electron-donating substituent, stereoselectivity is improved. It is preferably a 2,4-dimethoxybenzoyl group.

Uridine derivative 18 obtained by the Pummerer reaction can be converted to 4'-thiouridine 19 by conducting deprotection using ammonium fluoride and methylamine. Then, 4'-thio UTP is synthesized from 4'-thiouridine 19.


HO
S
U
HO
OH
19
1) MMTrCl
pyridine
2) Ac2O
86%
MMTrO
AcO
OAc
20
80% AcOH
84%
21
Cl
23
P2O7 4-
1) I2, pyridine, H2O
2) NH3/MeOH
24
4 steps 55%

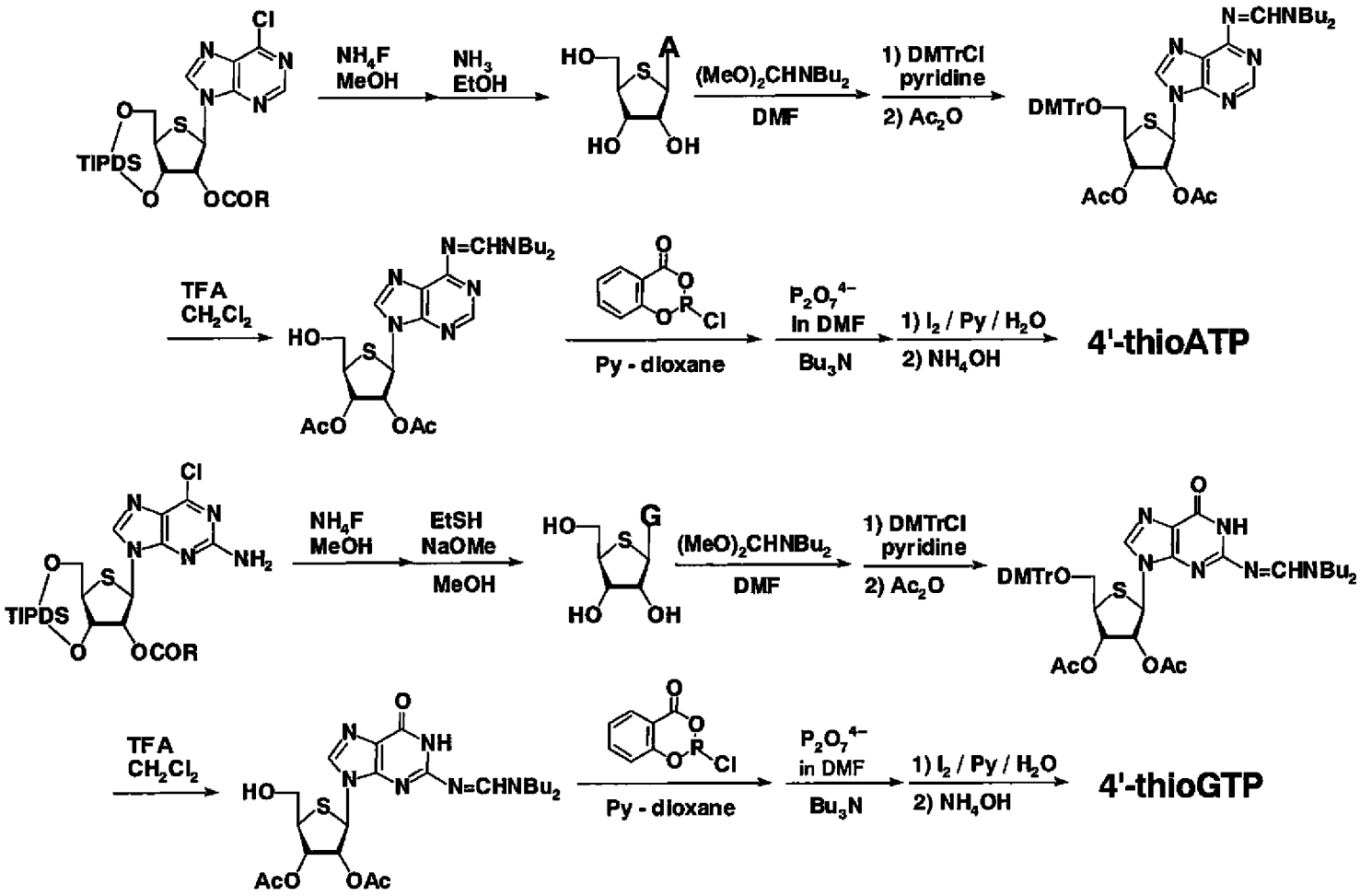
The 5'-position of compound 19 is monomethoxytritylated, and the 2'- and 3'-positions thereof are acetylated to obtain compound 20. Subsequently, demonomethoxytritylation is conducted to obtain acetyl compound 21. From the resulting compound 21, 4'-thio UTP 24 is synthesized by the method of Eckstein et al. (Luding, J. and Eckstein, F. (1989) J. Org. Chem., 54, 631-635). That is, the compound is converted into intermediate 23 using salicyl phosphorochloridite, and this intermediate is treated with pyrophosphoric acid to form a cyclotriphosphite compound. Iodo-oxidation, hydrolysis and deacetylation are then conducted to obtain the desired product 4'-thio UTP.

4'-Thio CTP can be produced from 4'-thiocytidine by the same process as the foregoing 4'-thio UTP after protecting nucleobase with protecting group such as benzoyl.


Bz
C
O
S
TIPDS
O
OCOR
1) NH4F
MeOH
2) MeNH2
HO
C
S
HO
OH
1
Bz2O
MeOH
1) MMTrCl
pyridine
2) Ac2O
MMTrO
Bz
C
S
AcO
OAc
2
80% aq AcOH
HO
Bz
C
S
AcO
OAc
3
O
O
P
O
Cl
Py - dioxane
P2O7 4-
in DMF
Bu3N
1) I2 / Py / H2O
2) NH4OH
4'-thioCTP

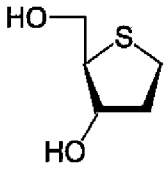
4'-Thio ITP can be produced from 4'-thiohypoxanthine by the same process as the foregoing 4'-thio UTP.

4'-Thio ATP and 4'-thio GTP can be produced by the following schemes.


Cl
NH4F
MeOH
NH3
EtOH
HO
A
HO
OH
(MeO)2CHNBu2
DMF
1) DMTrCl
pyridine
2) Ac2O
N=CHNBu2
DMTrO
TIPDS
OCOR
AcO
OAc
TFA
CH2Cl2
Py - dioxane
P2O7 4-
in DMF
Bu3N
1) I2 / Py / H2O
2) NH4OH
4'-thioATP
NH2
EtSH
NaOMe
MeOH
G
NH
O
4'-thioGTP

That is, after hydroxyl groups at the 2'- and 3'-positions and an amino group in the purine ring are appropriately protected, the compound is reacted with salicyl phosphorochloridite according to the method of Eckstein et al. (as mentioned above). The reaction product is then reacted with pyrophosphoric acid to obtain a cyclotriphosphite intermediate. Subsequently, iodo-oxidation, hydrolysis and deprotection are conducted to obtain 4'-thio ATP and 4'-thio GTP.

4'-Thio-2'-deoxyribonucleoside triphosphate can be produced in the same manner as 4'-thioribonucleoside triphosphate. A starting material, 4'-thio sugar:



is introduced a nucleobase at the 1-position using a Pummerer reaction and is reacted with salicyl phosphorochloridite. The resulting intermediate is reacted with pyrophosphoric acid, and iodo-oxidation, hydrolysis and deprotection are then conducted to obtain a desired product 4'-thio-2'-deoxyribonucleoside triphosphate.

The 4'-thionucleotides of the invention can be used as a substrate for a reaction of synthesizing DNA or RNA using a polymerase. To confirm that the 4'-thioribonucleotide of

the invention is recognized by RNA polymerase, the compound is contacted with RNA polymerase in the presence of an appropriate template, then determining whether the 4'-thioribonucleotide is incorporated into an oligomer. As demonstrated in figure 2, it has been found that the 4'-thio UTP synthesized according to the invention is recognized by T7 RNA polymerase and, like native nucleotides, incorporated into a synthetic oligomer chain.

In another aspect of the invention, the invention provides a process for producing an oligonucleotide using 4'-thionucleotides. The oligonucleotide can be produced by elongating an oligonucleotide chain with RNA or DNA synthetase, such as RNA polymerase or DNA polymerase, in the presence of the 4'-thionucleoside triphosphate of the invention. Various RNA polymerases derived from various organisms can be used as RNA synthetase. As DNA synthetase, DNA polymerase, reverse transcriptase, terminal deoxynucleotidyl transferase and the like derived from various organisms can be used. The conditions for the elongation reaction may vary with the polymerase used, and a skilled person can select appropriate reaction conditions. In the reaction, in addition to the 4'-thionucleoside triphosphate of the invention, native nucleoside triphosphates or modified nucleoside triphosphates known in the art may be present.

The oligonucleotide obtained by the process of the

invention can be used as a diagnostic or therapeutic agent and an research reagent in the form of an antisense oligonucleotide, a ribozyme, a primer, an aptamer, an antigene, RNAi, siRNA, a probe or the like. Preferably, the oligonucleotide of the invention has a length of from about 6 to about 50 nucleotides. In a more preferred embodiment of the invention, the oligonucleotide has a length of from about 12 to about 20 nucleotides. The oligonucleotide may contain modified sugars, for example, sugar having a substituent at the 2'-position. It may also contain a nucleic acid other than adenine, guanine, cytosine, thymine and uracil, for example, hypoxanthine, 5-alkylcytidine, 5-alkyluridine, 5-halouridine, 6-azapyrimidine or 6-alkylpyrimidine. Further, it may contain an inter-nucleoside linkage other than a phosphodiester, for example, a phosphorothioate linkage.

The oligonucleotide of the invention is appropriately used in vitro and in vivo because it has high degree of nuclease resistance and heat stability. It is especially useful in gene therapy.

Examples

The invention is illustrated more specifically below by referring to Examples. However, these Examples do not limit the scope of the invention.

In Examples, the following abbreviations are used.

Bz	benzoyl
DMAP	4-dimethylaminopyridine
DMBz	dimethoxybenzoyl
MMTr	4-methoxytrityl
Ms	methanesulfonyl
Tf	trifluoroethanesulfonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIPDS	1,1,3,3-tetraisopropyldicyloxane-1,3-diyl
TMS	trimethylsilyl
Ts	p-toluenesulfonyl

Example 1

Synthesis of 2,3,5-tri-O-p-methoxybenzyl-D-ribitol (3)

D-ribose (60 g, 0.4 mol) was dissolved in allyl alcohol (1.8 L, 26.4 mol), and conc. sulfuric acid (6.4 mL, 0.12 mol) was added at 0°C. Then, the solution was stirred overhead at room temperature. Subsequently, the reaction solution was neutralized by addition of sodium bicarbonate, and filtered with Celite. The filtrate was vacuum-dried by distilling off the solvent in vacuo to obtain a yellow oil residue. The residue dissolved in DMF (300 mL) was then cannulated in a THF (700 mL) solution of sodium hydride (64 g, 1.6 mol) at 0°C over a period of 3 hours. The reaction solution was returned again to room temperature, and stirred for 4 hours. Thereafter,

p-methoxybenzyl chloride (190 mL, 1.4 mol) was added dropwise at 0°C at a rate of 10 mL/15 min. When 100 mL was added dropwise, the solution was returned to room temperature, and the dropwise addition was carefully continued while observing a condition of H_2 occurrence. After completion of the dropwise addition, the solution was stirred at room temperature. After 13 hours, sodium hydride (4.0 g, 0.1 mol) and p-methoxybenzyl chloride (30 mL, 0.22 mol) were added, and the mixture was stirred for 24 hours. The reaction solution was neutralized with a saturated ammonium chloride aqueous solution. The solution was then diluted with ethyl acetate, and separated with water. The organic layer was washed with water (x3) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. Then, after cotton plug filtration, the solvent was distilled off, and the resulting brown residue was vacuum-dried. The product was dissolved in chloroform (1.2 L), and oxygen was included. Water (800 mL) was added thereto, and palladium chloride (21.2 g, 0.12 mol) was added. The solution was stirred overhead at 50°C. After 9 hours, palladium chloride (7.0 g, 0.04 mol) was added. After 28 hours, the reaction solution was returned to room temperature, filtered with Celite, concentrated, then diluted with ethyl acetate, and separated with water. The organic layer was washed with water (x2) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. Then, after

cotton plug filtration, the solvent was distilled off, and the resulting brown residue was vacuum-dried. The residue was dried, and then dissolved in methanol (1.2 L). Sodium borohydride (30.3 g, 0.8 mol) was added in an argon atmosphere at 0°C, and the solution was stirred for 20 minutes. The solution was returned to room temperature, and stirred. After 1 hour, sodium borohydride (7.8 g, 0.2 mol) was added at 0°C. The solution was then returned to room temperature, and stirred. After 1.5 hours, the solvent of the reaction solution was distilled off, and the residue was azeotroped twice with methanol. The resulting residue was dissolved in ethyl acetate, and the solution was separated with water. The organic layer was washed with water (x2) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. Then, after cotton plug filtration, the solvent was distilled off, and the residue was purified by silica gel chromatography (hexane:AcOEt = 5:1 -> 1:1) to obtain compound 3 (162.4 g, 79%) as colorless transparent oil.

^{1}H NMR (270 MHz, $CDCl_3$) δ: 7.30-6.81 (m, 12H, Ar), 4.66-4.40 (m, 6H, CH_2), 3.94-3.53 (m, 16H, H-1, 2, 3, 4, 5, MeOx3), 2.71 (br s, 1H, 4-OH), 2.36 (br s, 1H, 1-OH).

Example 2

Synthesis of

1,4-anhydro-2,3,5-tri-O-p-methoxybenzyl-4-thio-D-ribitol

(5)

Mesyl chloride (122 mL, 1.6 mol) was added to a pyridine solution (900 mL) of compound 3 (162 g, 0.32 mol) in an argon atmosphere at 0°C, and the solution was stirred at the same temperature for 30 minutes. Ice was then added to the reaction solution, and the mixture was stirred for 20 minutes. The reaction solution was diluted with ethyl acetate, and separated with water. The organic layer was washed with a saturated sodium hydrogencarbonate aqueous solution (x2) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. After cotton plug filtration, the solvent was distilled off in vacuo, and the residue was azeotroped three times with toluene. The residue was vacuum-dried, and then dissolved in MEK (1L) in an argon atmosphere. Lithium bromide (278 g, 3.2 mol) was added at room temperature, and the mixture was heat-refluxed. After 12 hours, the reaction solution was returned to room temperature, diluted with ethyl acetate, and separated with water. The organic layer was washed with water (x2) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. After cotton plug filtration, the solvent was distilled off in vacuo, and the resulting residue was vacuum-dried, and then dissolved in DMF (1 L) in an argon atmosphere. Sodium sulfide 9-hydrate (92.2 g, 0.38 mol) was added at room temperature, and the mixture was stirred at 100°C for 30 minutes. Subsequently, the reaction solution was

returned to room temperature, diluted with ethyl acetate, and separated with water. The organic layer was washed with water (x2) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. After cotton plug filtration, the solvent was distilled off in vacuo, and the resulting residue was coarsely purified by silica gel chromatography (hexane:AcOEt = 10:1 -> 1:1), and then recrystallized from hexane and ethyl acetate. Compound 5 (69.1 g, 42%) was obtained as a white crystal.

^{1}H NMR (270 MHz, $CDCl_3$) δ: 7.26-6.81 (m, 12H, Ar), 4.52-4.38 (m, 6H, CH_2), 4.01-3.95 (m, 1H, H-2), 3.91 (t, 1H, H-3, $J_{3,2}$ =4.0, $J_{3,4}$ =4.0 Hz), 3.80 (s, 9H, MeOx3), 3.66-3.60 (m, 1H, H-4), 3.45-3.40 (m, 2H, H-5a, H-5b), 3.02 (dd, 1H, H-1a, $J_{1a,1b}$= 10.6, $J_{1a,2}$ =6.5 Hz), 2.87 (dd, 1H, H-1b, $J_{1b,1a}$ =10.6, $J_{1b,2}$ =5.2 Hz).

Example 3

Synthesis of

1,4-anhydro-2-O-(4-methoxybenzoyl)-3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-4-thio-D-ribitol (13b)

4-Methoxybenzoyl chloride (723 μL, 5.1 mmol) was added to a pyridine solution (15 mL) of compound 12 (997 mg, 2.5 mmol) in an argon atmosphere at 0°C, and the mixture was stirred at room temperature for 18.5 hours. Ice was added to the reaction solution, and the mixture was stirred for 10 minutes. Then,

the solution was diluted with ethyl acetate, and separated with water. The organic layer was washed with a saturated sodium hydrogencarbonate aqueous solution (x3) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. After cotton plug filtration, the solvent was distilled off in vacuo, and the residue was azeotroped three times with toluene. The resulting residue was purified by silica gel chromatography (hexane:AcOEt = 50:1 -> 35:1) to obtain compound 13 (1.3 g, 96%) as colorless oil.

FAB-LRMS m/z 527 (MH^+).

FAB-HLRS Calcd for $C_{25}H_{42}O_6SSi_2$ (MH^+): 527.2319. Found: 527.2311.

^1H NMR (400 MHz, $CDCl_3$) δ: 7.95-6.61 (m, 4H, Ar), 5.71-5.69 (m, 1H, H-2), 4.35 (dd, 1H, H-3 $J_{3,2}$ =3.5, $J_{3,4}$ =9.4 Hz), 4.12 (dd, 1H, H-5a, $J_{5a,4}$ =2.9, $J_{5a,5b}$ =12.6 Hz), 3.98 (dd, 1H, H-5b, $J_{5b,4}$ =3.2, $J_{5b,5a}$ =12.6 Hz), 3.69 (m, 1H, H-4), 3.24 (dd, 1H, H-1β, $J_{1\beta,2}$= 3.2, $J_{1\beta,1\alpha}$ =12.6 Hz), 3.04 (m, 6H, Me_2N), 2.91 (d, 1H, H-1α, $J_{1\alpha,1\beta}$ =12.6 Hz), 1.13-0.87 (m, 28H, TIPDS).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 166.40, 153.59, 131.73, 117.34, 110.94, 110.81, 75.95, 75.21, 60.16, 51.29, 40.44, 31.60, 17.84, 17.77, 17.70, 17.66, 17.52, 17.45, 13.87, 13.75, 13.15, 13.09.

Example 4

Synthesis of

1,4-anhydro-2-O-(4-dimethylaminobenzoyl)-3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-4-thio-D-ribitol (13c)

Thionyl chloride (933 μL, 12.8 mmol) was added to a methylene chloride solution (40 mL) of 4-dimethylaminobenzoic acid (1.1 g, 6.4 mmol) in an argon atmosphere, and the mixture was heated to reflux with stirring for 2 hours. The temperature was returned to room temperature, and the solvent was distilled off in vacuo. The resulting residue (533 mg) was added to a pyridine solution (5 mL) of compound 12 (375 mg, 0.96 mmol) at 0°C in an argon atmosphere, and the mixture was stirred at room temperature for 8 hours. After 8 hours, the foregoing residue (355 mg) was added again. After 21 hours, pyridine (5 mL) was added, and the mixture was heated at 50°C for 6 hours. Ice was added to the reaction solution, and the mixture was stirred for 10 minutes. The reaction solution was then diluted with ethyl acetate, and separated with water. The organic layer was washed with a saturated sodium hydrogencarbonate aqueous solution (x3) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. After cotton plug filtration, the solvent was distilled off in vacuo, and azeotroped three times with toluene. The resulting residue was purified by silica gel chromatography (hexane:AcOEt = 50:1 -> 20:1) to obtain compound 13c (476 mg, 92%) as colorless oil.

FAB-LRMS m/z 540(MH^+).

FAB-HLRS Calcd for $C_{26}H_{45}NO_5SSi_2$ (MH^+): 540.2635. Found: 540.2637.

^1H NMR (400 MHz, $CDCl_3$) δ: 7.95-6.61 (m, 4H, Ar), 5.71-5.69 (m, 1H, H-2), 4.35 (dd, 1H, H-3 $J_{3,2}$ =3.5, $J_{3,4}$ =9.4 Hz), 4.12 (dd, 1H, H-5a, $J_{5a,4}$ =2.9, $J_{5a,5b}$ =12.6 Hz), 3.98 (dd, 1H, H-5b, $J_{5b,4}$ =3.5, $J_{5b,5a}$ =12.6 Hz), 3.69 (m, 1H, H-4), 3.24 (dd, 1H, H-1β, $J_{1\beta,2}$= 3.2, $J_{1\beta,1\alpha}$ =12.6 Hz), 3.04 (m, 6H, Me_2N) 2.91 (d, 1H, H-1α, $J_{1\alpha,1\beta}$ =12.6 Hz), 1.13-0.87 (m, 28H, TIPDS).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 166.40, 153.59, 131.73, 117.34, 110.94, 110.81, 75.95, 75.21, 60.16, 50.03, 40.44, 31.60, 17.84, 17.77, 17.71, 17.67, 17.52, 17.45, 13.87, 13.75, 13.15, 13.09.

Example 5

Synthesis of 1,4-anhydro-2-O-(4-methoxybenzoyl)-3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-4-thio-D-ribitol-1-oxide (14b)

Ozone gas was bubbled in a methylene chloride solution (20 mL) of compound 13b (1.1 g, 2.3 mmol) at -78°C for 20 minutes. Argon was bubbled in the reaction solution until the ozone odor disappeared, and the temperature was then raised to room temperature. After the solvent was distilled off in vacuo, the residue was purified by silica gel chromatography (hexane:AcOEt = 4:1 -> 1:3) to obtain compound 14b (1.0 g, 82%) as a colorless sticky product.

FAB-LRMS m/z 543 (MH^+).

FAB-HLRS Calcd for $C_{25}H_{42}O_7SSi_2$ (MH^+): 543.2253. Found: 543.2251.

^1H NMR (400 MHz, $CDCl_3$) δ: 8.06-6.90 (m, 4H, Ar), 5.84-5.82 (m, 1H, H-2), 4.61 (d, 1H, H-5a, $J_{5a,\ 5b}$ =12.9 Hz), 4.23 (dd, 1H, H-5b, $J_{5b,\ 4}$ =2.9, $J_{5b,\ 5a}$ =12.9 Hz), 4.17 (dd, 1H, H-3 $J_{3,\ 2}$ =3.5, $J_{3,\ 4}$ =12.0 Hz), 3.86 (s, 3H, MeO), 3.61(dd, 1H, H-1β, $J_{1\beta,\ 2}$= 5.3, $J_{1\beta,\ 1\alpha}$ =15.5 Hz), 3.48 (dd, 1H, H-4, $J_{4,\ 5b}$ =2.1, $J_{4,\ 3}$ =12.0 Hz), 2.93 (d, 1H, H-1α, $J_{1\alpha,\ 1\beta}$ =15.5 Hz), 1.09-0.87 (m, 28H, TIPDS).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 165.42, 163.84, 132.20, 122.24, 113.96, 73.31, 73.15, 68.48, 55.79, 55.75, 54.59, 17.72, 17.64, 17.58, 17.54, 17.53, 17.49, 17.31, 17.30, 13.81, 13.51, 13.02, 13.00.

Example 6

1-[2-O-(4-methoxytrityl)-3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-4'-thio-β-D-ribofuranosyl]uracil (15b)

Triethylamine (1.0 mL, 7.3 mmol) and TMSOTf (2.6 mL, 14.6 mmol) were added to a toluene suspension (20 mL) of uracil (408 mg) in an argon atmosphere at room temperature, and the reaction solution was stirred until a two-layer solution was formed. Methylene chloride (10 mL) was added to this reaction solution to form a monolayer solution. This reaction solution was added dropwise to a methylene chloride solution (20 mL) of compound

14b (987 mg, 1.8 mmol) at room temperature over a period of 15 minutes. Subsequently, a toluene solution (10 mL) of triethylamine (1.0 mL, 7.3 mmol) was added dropwise at room temperature over a period of 5 minutes. Water was added to the reaction solution, and the mixture was stirred for 10 minutes. The solution was then diluted with ethyl acetate, and separated with water. The organic layer was washed with a saturated sodium hydrogencarbonate aqueous solution (x3) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. After cotton plug filtration, the solvent was distilled off in vacuo. The residue was purified by silica gel chromatography (hexane:AcOEt = 49:1 -> 1:1) to obtain compound 15b (904 mg, 77%) as a white foam.

FAB-LRMS m/z 637 (MH^+).

FAB-HLRS Calcd for $C_{29}H_{44}N_2O_8SSi_2$ (MH^+): 637.2435. Found: 637.2435.

^1H NMR (400 MHz, $CDCl_3$) δ: 9.28 (br s, 1H, NH), 8.22 (dd, 1H,H-6, $J_{6,5}$ =8.2), 8.02-6.94 (m, 4H, Ar), 6.01 (s, 1H, H-1'), 5.76 (dd, 1H, H-5, $J_{5,6}$ =8.2, $J_{5,NH}$ =2.1 Hz), 5.62 (d, 1H, H-2', $J_{2',3'}$ =3.5), 4.45 (dd, 1H, H-3' $J_{3',2'}$ =3.5, $J_{3',4'}$ =9.4 Hz), 4.18-4.06 (m, 2H, H-5'a, H-5'b), 3.87 (s, 3H, MeO), 3.73-3.71 (m, 1H, H-4'), 1.15-0.86 (m, 28H, TIPDS).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 164.01, 163.38, 163.10, 150.13, 140.653, 131.79, 121.73, 113.53, 102.19, 102.17, 78.06, 71.24, 62.54, 57.92, 55.40, 55.39, 50.69, 17.47, 17.36, 17.34, 17.27,

16.99, 16.96, 16.83, 16.79, 13.34, 13.18, 13.10, 13.48.

Example 7

1-[5-O-(4-methoxytrityl)-2,3-O-diacetyl-4'-thio-β-D-ribofuranosyl]uracil (20)

In an argon atmosphere, 4-methoxytrityl chloride (232 mg, 0.75 mmol) was added to a pyridine (4 mL) solution of 1-(4-thio-β-D-ribofuranosyl)uracil (131 mg, 0.5 mmol; produced by deprotecting compound 15 with NH_4F/MeOH and $MeNH_2$/MeOH), and the mixture was stirred at room temperature for 14 hours. Acetic anhydride (188 μL, 2 mmol) and DMAP (5 mg, 0.05 mmol) were added in an argon atmosphere, and the mixture was stirred at room temperature for 2 hours. Methanol was added, and the solution was stirred for 30 minutes. The solvent was distilled off in vacuo from the reaction solution. The residue was dissolved in ethyl acetate, and the solution was separated with water. The organic layer was washed with a saturated sodium hydrogencarbonate aqueous solution (x3) and with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. After cotton plug filtration, the solvent was distilled off in vacuo. The residue was purified by silica gel chromatography (hexane:AcOEt = 2:1 -> 1:1) to obtain compound 20 (214 mg, 70%) as a colorless transparent solid. ^1H NMR (270 MHz, $CDCl_3$) δ: 8.07 (br s, 1H, NH), 7.77 (d, 1H,H-6, $J_{6,5}$ =7.9), 7.47-6.85 (m, 14H, MMTr), 6.37 (d, 1H, H-1', $J_{1'}$,

$_{2'}$=7.3), 5.68 (dd, 1H, H-2', $J_{2',1'}$=7.3, $J_{2',3'}$=4.0), 5.54-5.51 (m, 1H, H-3'), 5.62 (dd, 1H, H-5, $J_{5,6}$=7.9, $J_{5,NH}$=2.6Hz), 3.81 (s, 3H, MeO), 3.62-3.53 (m, 2H, H-5'a, H-4'), 3.40-3.35 (m, 1H, H-5'b), 2.12-2.00 (m, 6H, AcOx2).

Example 8

1-(2,3-O-diacetyl-4'-thio-β-D-ribofuranosyl)uracil (21)

Compound 20 (199 mg, 0.32 mmol) was dissolved in a 80% acetic acid aqueous solution, and the mixture was stirred at room temperature for 11 hours. The reaction solution was added dropwise to a saturated sodium hydrogencarbonate aqueous solution, and the mixture was separated with ethyl acetate. The aqueous layer was extracted seven times with chloroform. The organic layer was washed with a saturated sodium chloride aqueous solution (x1), and dried over Na_2SO_4. After cotton plug filtration, the solvent was distilled off in vacuo. The residue was purified by silica gel chromatography (hexane:AcOEt = 2:1 -> AcOEt) to obtain compound 21 (93 mg, 84%) as a white foam.

^{1}H NMR (270 MHz, $CDCl_3$) δ: 8.23 (br s, 1H, NH), 8.08 (d, 1H,H-6, $J_{6,5}$=7.9), 6.36 (d, 1H, H-1', $J_{1',2'}$=7.3), 5.85 (m, 1H, H-5), 5.69 (dd, 1H, H-2', $J_{2',1'}$=7.3, $J_{2',3'}$=4.0), 5.49 (m, 1H, H-3'), 4.16-4.04 (m, 1H, H-5'a), 3.86-3.82 (m, 1H, H-5'b), 3.56-3.55 (m, 1H, H-4'), 2.42 (br s, 1H, OH), 2.17 (s, 3H, AcO), 2.06 (s, 3H, AcO)

Example 9

1-(4'-thio-β-D-ribofuranosyl)uracil 5'-triphosphate(24)

Compound 21 (89 mg, 0.26 mmol) was azeotroped with pyridine, and dissolved in pyridine (260 μL). Dioxane (780 μL) was added in an argon atmosphere, and the solution was stirred at room temperature. A dioxane solution of salicyl phosphorochloridite (58 mg, 2.9 mmol) was added, and the mixture was stirred for 10 minutes. A DMF solution (780 μL) of 0.5 M bisbutylammonium pyrophosphate was added, and butylamine (260 μL) was quickly added, followed by stirring for 10 minutes. 1% Iodine (5 mL) was added in pyridine/water (98/2, v/v), and the mixture was stirred for 5 minutes. Several drops of 5% sodium hydrogensulfite aqueous solution were added, and the mixture was stirred for 40 minutes. Aqueous ammonia (8 mL) was added to the reaction solution, and the mixture was stirred for 3.5 hours. The solvent was distilled off in vacuo. The residue was dissolved in 300 mL of water, and the solution was purified by ion exchange chromatography (H_2O -> 1N TEAB), and then purified via a salt exchange column (H^+ type). The solvent was distilled off in vacuo. The residue was dissolved in 5 mL of water, and the solution was purified via a salt exchange column (Na^+ type). The solvent was distilled off in vacuo. Compound 24 (80 mg, 55%) was obtained as pale yellow oil.

FAB-LRMS m/z 565 ($M-3Na^+$).

^{31}P-NMR(108 MHz, D_2O) δ: -10.27 (d, J = 20 Hz), -10.56 (d, J = 20 Hz), -21.27 (t, J = 20 Hz).

Example 10

Incorporation of 4'-thio UTP into RNA chain with T7 RNA polymerase

Incorporation experiment using T7 RNA polymerase was performed with 4'-thio UTP obtained in Example 8. In this experiment, double-stranded DNA containing T7 promoter sequence was used (Fig. 1). When all of the nucleotides ATP, GTP, CTP and UTP are present, a complementary 26mer RNA shown in the figure ought to be synthesized.

The reaction was conducted in 20 μL of a solution containing 40 mM Tris-HCl with PH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.4 mM NTPs, 17 nM [γ-^{32}P]GTP, 2.0 μM template DNA and 100 U T7 RNA polymerase. NTPs were (1) GTP, (2) GTP + ATP, (3) GTP + ATP + CTP, (4) GTP + ATP + CTP + UTP, and (5) GTP + ATP + CTP + 4'-thio UTP, respectively. The reaction solution was incubated at 37°C for 3 hours, and the reaction was stopped. Subsequently, electrophoresis was performed with 20% modified polyacrylamide gel (30 x 40 x 0.05 cm, 1800 V, 1 hour, 1 x TEB), and analysed by autoradiography.

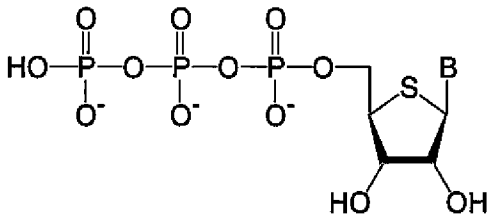
The results are shown in Fig. 8. In lane 1, bands corresponding to 2mer and 3mer of G and a rudder were observed.

Fig. 1 shows an outline of the experiment of incorporating 4'-thio UTP using T7 RNA polymerase.

Fig. 2 is electropherogram showing results of the experiment of incorporating 4'-thio UTP using T7 RNA polymerase.

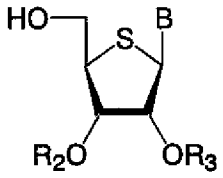
Abstract

Disclosed is a compound of formula I:



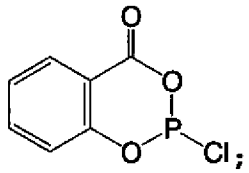
[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, uracil and hypoxanthine], and a method for synthesizing a compound of formula I:

said method comprising reacting a compound of formula III:



[wherein B is a nucleobase selected from the group consisting of adenine, guanine, cytosine, uracil and hypoxanthine, and each of R_2 and R_3 is, independently a protecting group of a hydroxyl group]

with a compound of formula IV:



reacting the resulting intermediate with pyrophosphoric acid; and

conducting iodo-oxidation, hydrolysis and deprotection to obtain the compound of formula I.

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Metabolism of 4′-Thio-β-D-arabinofuranosylcytosine in CEM Cells

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ABSTRACT. Because of the excellent *in vivo* activity of 4′-thio-β-D-arabinofuranosylcytosine (T-araC) against a variety of human solid tumors, we have studied its metabolism in CEM cells to determine how the biochemical pharmacology of this compound differs from that of β-D-arabinofuranosylcytosine (araC). Although there were many quantitative differences in the metabolism of T-araC and araC, the basic mechanism of action of T-araC was similar to that of araC: it was phosphorylated to T-araC-5′-triphosphate (T-araCTP) and inhibited DNA synthesis. The major differences between these two compounds were: (i) T-araC was phosphorylated to active metabolites at 1% the rate of araC; (ii) T-araCTP was 10- to 20-fold more potent as an inhibitor of DNA synthesis than was the 5′-triphosphate of araC (araCTP); (iii) the half-life of T-araCTP was twice that of araCTP; (iv) the catalytic efficiency of T-araC with cytidine deaminase was 10% that of araC; and (v) the 5′-monophosphate of araC was a better substrate for deoxycytidine 5′-monophosphate deaminase than was the 5′-monophosphate of T-araC. Of these differences in the metabolism of these two compounds, we propose that the prolonged retention of T-araCTP is a major factor contributing to the activity of T-araC against solid tumors. The data in this study represent another example of how relatively small structural changes in nucleoside analogs can profoundly affect the biochemical activity. BIOCHEM PHARMACOL 60;12:1925–1932, 2000. © 2000 Elsevier Science Inc.

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In the last few years, we have designed and synthesized many 4′-thionucleoside analogs in our drug development program. The most promising antitumor compound that we have discovered in this series is T-araC§ [1, 2], which is structurally related to araC (Fig. 1), an agent currently used in the treatment of acute myelogenous leukemia. T-araC was first reported by Whistler *et al.* in 1971 [3], and although it was determined to be cytotoxic to KB cells (IC_{50} of 0.42 μM), no further biological studies were reported, presumably due to the lack of compound and the difficulty of the synthetic route used to make it. Our synthetic procedures have allowed us to generate large amounts of T-araC and test it in various animal tumor models. Of particular interest is the broad spectrum of activity that was observed with T-araC. Unlike araC, T-araC has demonstrated excellent *in vivo* antitumor activity against many solid tumors, including human colon, non-small cell lung, prostate, and renal tumor xenografts, and it is highly effective when administered once daily during the treatment period, whereas multiple daily doses of araC are necessary to obtain marginal antitumor activity [2].

The only structural difference between T-araC and araC is the replacement of the oxygen atom in the arabinose ring by a sulfur atom. The reasons why this relatively minor structural difference resulted in profound differences in antitumor activity are of great interest, and a complete understanding of the differences in the biochemical pharmacology of these two agents could give insight into the characteristics of nucleoside analogs that are required for activity against solid tumors. Therefore, we have initiated studies to characterize the biochemical pharmacology of T-araC to determine how it and its metabolites interact with the various enzymes associated with dCyd metabolism. For comparison purposes, we also have evaluated the metabolism of araC, T-dCyd, and dCyd. These studies, which are the first to characterize the metabolism of

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§ *Abbreviations:* araC, β-D-arabinofuranosylcytosine; araCMP, 5′-monophosphate of araC; araCTP, 5′-triphosphate of araC; araU, β-D-arabinofuranosyluracil; araUMP, 5′-monophosphate of araU; Cyd, cytidine; Cyt, cytosine; dCyd, 2′-deoxycytidine; dThd, thymidine; dUrd, 2′-deoxyuridine; F-dUrd, 5-fluoro-2′-deoxyuridine; IC_{50}, concentration of compound that inhibits cell growth by 50%; T-araC, 4′-thio-β-D-arabinofuranosylcytosine; T-araCMP, 5′-monophosphate of T-araC; T-araCTP, 5′-triphosphate of T-araC; T-araU, 4′-thio-β-D-arabinofuranosyluracil; T-araUMP, 5′-monophosphate of T-araU; T-dCyd, 4′-thio-2′-deoxycytidine; T-dCMP, 5′-monophosphate of T-dCyd; T-dCTP, 5′-triphosphate of T-dCyd; T-dThd, 4′-thiothymidine; T-dUrd, 4′-thio-2′-deoxyuridine; T-dUMP, 5′-monophosphate of T-dUrd; and Urd, uridine.

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T-araC, T-dCyd, T-[illegible]